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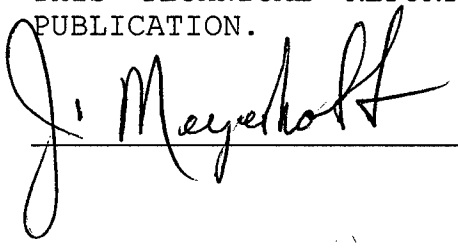

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13. ABSTRACT *(Maximum 200 words)*

Neurotrophic factors, including brain-derived neurotrophic factor (BDNF), may provide neuroprotection to the brain following injury. Previous studies have demonstrated that mRNA levels of BDNF and its receptor (trkB) are altered following experimental brain injury in the rat. The present study investigated whether these injury-induced alterations in BDNF and trkB mRNA levels lead to subsequent alterations in protein levels. In addition, activation of the BDNF/trkB signal transduction cascade was evaluated by measuring protein levels of a major downstream effector, extracellular signal regulated kinase, in its inactive (ERK) and activated form (MAPK). Protein levels of BDNF, trkB, ERK, and MAPK were investigated in the cortex and hippocampus between 3 h and 5 d after lateral fluid percussion brain injury in the rat. BDNF protein levels were significantly increased in the bilateral hippocampus 48 h after injury. No significant alterations were observed in trkB, ERK, or MAPK protein levels after injury in either the cortex or hippocampus. These data suggest that the endogenous alterations in BDNF that occur after experimental brain injury are not activating the ERK pathway, but involvement of other pathways cannot be excluded.

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Ramona Hicks 1-10-99

PI - Signature Date

4. Table of Contents

5. Introduction

The long-term objective of this research study is to identify treatments that will improve functional outcomes after traumatic brain injury. One potential treatment to attenuate deficits associated with traumatic brain injury involves the administration of neurotrophic factors (NTF). NTFs are a family of structurally related polypeptides that may mediate a protective response following experimental brain injury (Hefti et al., 1989; Mattson and Scheff, 1994). Members of the NTF family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin 4/5, and neurotrophin 6 (Thoenen, 1991). NTFs are believed to promote cell survival through their interactions with a common low-affinity receptor, the p75 receptor (Rabizadeh et al., 1993; Carter et al., 1996). Differences in NTF actions are mediated through their interactions with specific high-affinity tyrosine kinase (trk) receptors (Meakin and Shooter, 1992; Barbacid, 1994).

Numerous investigations are currently underway to elaborate the specific actions of the various NTFs. In brief, NGF/trkA interactions are believed to be important for cell survival during development and following injury (for review, see Mattson and Scheff, 1994), and to promote sprouting and regeneration of connections of cholinergic neurons (Dekker et al., 1994; Tuszynski et al., 1990). In an animal model of TBI, infusion of NGF into the lesion improved cognitive function without overt effects on neuropathology (Sinson et al, 1995). BDNF is the most abundantly expressed NTF in the mature central nervous system (Hofer et al., 1990) and supports the survival of many types of neurons (Lindsay, 1993). BDNF/trkB interactions appear to be neuroprotective following various forms of brain injury (Beck et al., 1994; Cheng & Mattson 1994; Hayes et al., 1995; Kindy 1993; Kubo et al., 1995; Schabitz et al., 1997; Skaper et al., 1993). However, this is controversial as a few studies have implicated BDNF as a contributing factor to neuronal degeneration (Koh et al., 1995; Rudge et al., 1998). BDNF also appears to be important for learning and memory, including having a role in long-term potentiation (Dragonow et al., 1997; Figurov et al., 1996; Korte et al., 1996) and activity-dependent neuroplasticity (Gall 1992; Marty et al., 1997; Rocamora et al., 1996). NT-3/trkC signal transduction has also been found to have neuroprotective effects in some models of neuronal injury (Hagg 1997; Lindsay 1993). Less is known about the other NTF/trk interactions, but they may also contribute to neuroprotection for various regions of the brain or CNS disorders (Mattson & Scheff, 1994; Lindsay 1993).

These studies support the hypothesis that NTFs may influence outcomes following brain injury. However numerous studies have also demonstrated that brain injury alters NTF gene expression (Gall 1993; Hicks et al., 1997, 1998; Isackson et al., 1991; Yang et al., 1996). Studies undertaken in year I of this project demonstrated that there are region- and time-dependent alterations in BNDF, trkB, and NT-3 mRNA levels between 1 and 72 h after both mild and moderate levels of lateral fluid percussion brain injury (FPI) compared to sham-injured control animals (Hicks et al., 1997, 1998, Appendices I-II). The effects of these alterations on NTF gene expression are unknown. The purpose of the second year of this grant was to investigate whether there are time-dependent alterations in BDNF/trkB signal transduction pathways following FPI. In

other words, are the alterations in BDNF and trkB mRNA levels associated with similar alterations in protein levels and subsequent activation of BDNF/trkB signaling pathways? (Fig. 1).

6. Body

Methods

Animal surgery. All surgical procedures were done under sterile conditions. The procedure for the lateral FPI has been described in detail in a previous publication (McIntosh et al., 1989). Briefly, male Sprague-Dawley rats (300-350 g) were anesthetized with sodium pentobarbital (60 mg/Kg) and placed in a stereotaxic device. Animals were given a craniotomy centered between bregma and lambda, just lateral to the sagittal suture. The dura was checked to insure that it remained intact. After the craniotomy was trephined into the skull, a small stainless steel screw was placed 1 mm rostral to bregma and 2 mm lateral to the sagittal suture. A rigid plastic tube (modified Leur-Loc syringe hub, 4.9 mm outer diameter) was placed over the exposed dura and bonded with cyanoacrylate adhesive. Dental acrylic was then poured around the plastic tube and around the stainless steel screws. After the acrylic had hardened, the tube was filled with isotonic saline and attached to the male Leur-Loc fitting at the end of the injury device. The animals were subjected to a FPI of moderate severity (2.0 atm). Following the FPI, the dental acrylic and plastic tube were removed with a hemostat. The dura was checked once again to insure that it was intact and the scalp was closed with wound clips. Animals were allowed to survive from 3h to 5d depending upon group designation. A control group of animals also received a sham-injury.

Tissue processing. Animals from each group were deeply anesthetized under sodium pentobarbital anesthesia (85mg/kg) prior to euthanasia. For enzyme-linked immunosorbent assays (ELISA) and Western blot procedures, animals were decapitated, the brains rapidly removed, and dissected into the following brain regions: injured cortex, opposite cortex, ipsilateral and contralateral hippocampus. The tissue was weighed and then snaps frozen in liquid nitrogen. Tissue was later homogenized in lysis buffers, centrifuged for 30 min, and the supernatant was aliquoted into 100 ul samples. For Western blots, μg of protein/ml tissue sample was determined by spectrophotometric analysis. For histological procedures, animals were perfused with 0.9% saline followed by fixation with formalin. The brains were removed and post-fixed in formalin overnight, and then transferred into a 15% sucrose in 0.8 M phosphate buffered saline (PBS) solution and stored at 4° C.

ELISA procedure. Protein levels of BDNF and NT-3 were measured for the cortex and hippocampus using ELISA techniques (Ostermann-Latif et al., 1993). ELISA kits for BNDF and NT-3 (Promega) were used in these studies and the procedures described with the kits were followed. Briefly, microwell plates were coated with either a monoclonal antibody for BDNF or NT-3 and incubated overnight at room temperature. The next day, the plates were washed, incubated with a blocking solution containing

normal serum for 1 h, and incubated with protein standards for BDNF or NT-3 or sample tissue for 2-6 hours. The wells were washed, incubated with a polyclonal antibody for either BDNF or NT-3 (incubation periods range from 2 h – overnight depending on the protein), washed, incubated with an appropriate secondary antibody conjugated to HRP for 2 h, washed, and developed in a 50:50 solution of TMB and peroxidase substrate for 15 min. The reaction was stopped by adding 100 µl of 1M phosphoric acid. Protein level measurements for each well were made with a color microplate reader.

To control for variability in loading the samples, standard amounts of protein were run in duplicate in amounts ranging from 0 – 500 pg/well. Sample tissue was measured at two different volumes (15 and 30 µl) and also run in duplicates. Mean values were calculated for each animal and expressed as ng/g tissue and also as a percentage of control (sham) values.

Western blot procedure. Levels of the full-length (trkB-145) and truncated (trkB-95) high-affinity receptor for BDNF, a downstream protein kinase called extracellular signal regulated kinase (ERK), and a phosphorylated form of ERK (MAPK) were measured after FPI. Total protein levels were measured following tissue homogenization in a lysis buffer with a spectrophotometer and then the tissue was stored at –80° until ready for use. For trkB (Transduction, 1:250), 60 µg of protein, for ERK, 20 µg of protein (Transduction, 1:10,000) and for MAPK (Promega, 1:20,000) 20 µg of protein were subjected to SDS-polyacrylamide gel electrophoresis (Jahn et al., 1984). Following separation, the proteins were transferred to nitrocellulose paper and then incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (anti-mouse IgG, Transduction Laboratories, 1:20,000; anti-rabbit IgG, Amersham, 1:5,000). Lastly they were incubated in a chemiluminescent solution (Pierce) and then exposed to hyperfilm (Amersham) to allow visualization of the blots. Pre-stained molecular weight standards (Bio-Rad) were also run on the gel to confirm that the immunoreactive band was at the appropriate molecular weight. The films were digitized onto a computer and optical densities of the blots were analyzed with gel plotting software (Image 1.60, NIH).

As a control for non-specific binding, additional protein samples were run on gels but incubation with the primary antibody was omitted. To evaluate the sensitivity of the Western blot procedure to detect differences in protein levels, known and graded amounts of protein from the same animal were run on the gels and analyzed for corresponding differences in optical densities.

BDNF injections. A few animals received BDNF after FPI as a preliminary investigation into the effects of time and method of delivery on neuropathology. Animals received either BDNF (Regeneron Pharmaceuticals, 10mg/kg, iv) or saline 10 min or 24 h after receiving a moderate FPI (n=2/group).

Histology. Brains were cut into 40 µm thick sections with a freezing microtome throughout the level of the lesion. Sections were stained with cresyl violet to evaluate the extent of neuronal injury. Cortical lesion volumes were determined by digitizing and analyzing brain regions with image processing software (Image 1.60, NIH). To measure

the lesion volume, brain sections were viewed under brightfield magnification and the area which contained necrotic or damaged tissue was carefully circumscribed. The volumes were determined using the Cavalieri method (Michel and Cruz-Orive, 1988).

Statistical analysis. Two-way ANOVAs followed by Bonferonni post-hoc analyses were used to analyze the effects of group and time and their interaction on protein levels in various regions of the brain. A Student's T-test was used to evaluate differences in lesion volume measurements between animals that received BDNF or saline after injury.

Results and Discussion

Neurotrophic Factor Protein Levels after FPI. BDNF protein levels in the injured cortex (IC) and opposite cortex (OC) were not significantly different compared to sham animals at either 6 h, 48 h, or 5 d post-FPI, although there was a trend toward a bilateral decrease at 48 h (Fig. 2). Previous studies demonstrated that BDNF mRNA was not altered in the IC until 72 h after FPI, at which time it was significantly decreased (Appendix I). The increase in BDNF protein in the IC at 5 d compared to 48 h is perplexing, as there is no evidence to suggest that the IC recovers over time. The most likely explanation, is that by 5 d much of the IC tissue has been cleared away. Attempts at dissecting out the IC at this time point may have resulted in a larger percentage of tissue in adjacent regions also being included in the samples. The decrease in BDNF in the OC at 48 h was also unexpected as no overt damage has been reported in this area previously, nor were alterations in BDNF mRNA observed (Appendix I).

BDNF protein levels were significantly increased at 48 h post-FPI in the bilateral hippocampus ($P < 0.003$; Fig. 3). This is in agreement with alterations in BDNF mRNA which were significantly increased in the bilateral hippocampus up to 72 h post-FPI (Hicks et al., 1997).

NT-3 protein levels were only measured in the hippocampus because cortical levels are very low under normal conditions. No significant differences in NT-3 protein levels were observed between injured and control animals, although there was a strong trend toward a decrease in protein at 6 h (Fig. 4). This trend toward an acute loss of NT-3 protein followed by recovery to control levels by 48 h is in general agreement with alterations reported in NT-3 mRNA (Hicks et al., 1997).

trkB Protein Levels after FPI. No significant changes were observed in either the full-length or the truncated form of trkB protein in the IC (Figs. 5 and 6). The full-length receptor, trkB-145 is the one that was analyzed for mRNA changes previously (Hicks et al., 1998), and levels were rapidly and progressively decreased after FPI. Why these changes in mRNA levels are not reflected in protein levels is unknown, but it is possible that the turnover of trkB protein is attenuated after FPI. Differences between mRNA and protein levels are not uncommon in the brain following various manipulations. The maintenance of trkB-95 levels after FPI may also be related to a decrease in turnover rate, as well as attributable to the gliosas which occurs following FPI (Cortez et al., 1989).

The truncated form of the trkB receptor has been observed in astrocytes, as well as in neurons, however, the full-length form is believed to be restricted to neuronal populations (Roback et al., 1995).

In the hippocampus, no significant changes were observed in either trkB-95 or trkB-145 at any of the post-FPI time points (Figs. 7 and 8). Although significant increases were observed in trkB mRNA in the dentate gyrus, this was not true for other subregions of the hippocampus (Hicks et al., 1998). Therefore, it is possible that these alterations in mRNA did not lead to detectable differences in protein levels following evaluation of the entire hippocampus. In culture, six days of chronic exposure to BDNF resulted in down-regulation of trkB-145 protein (Knusel et al., 1997). Although there was a trend toward a decrease in trkB-145 in the hippocampus at 96 h, we did not see significant down-regulation in response to acute increases in BDNF.

ERK and MAPK Protein Levels after FPI. Similar to BDNF and trkB, no significant alterations in ERK protein levels were observed in the IC or the IH after FPI (Figs. 9 and 10). Furthermore, no significant increases were seen in MAPK (the activated form of ERK) in the hippocampus (Fig. 11), despite an increase in BDNF protein levels (Fig. 3). Thus, it appears that acute increases in BDNF in the hippocampus following FPI do not lead to significant downstream activation of ERK. However, it is possible that other BDNF/trkB signal pathways are activated, i.e. the JNK pathway. Also it is noteworthy that chronic infusions of BDNF both in vitro (Roback et al., 1995) and in vivo in normal rats (Berhow et al., 1996) resulted in increased levels of MAPK. This suggests that the endogenous response of NTFs to FPI is transient and does not have pronounced downstream effects, but that administration of BDNF is capable of activating intracellular signaling pathways.

Effects of Injecting BDNF on Neuropathology. This preliminary study demonstrated that the lesion volume was significantly smaller ($P > 0.004$) in animals that received an intravenous injection of BDNF following FPI (Fig. 12). One of our major concerns with using BDNF as a pharmacological agent involves the difficulty in targeting it to the injured brain. Thus, in one animal, we injected BDNF 15 min after FPI and then measured protein levels in the IC, OC, IH, and OH 2 h after the injection. Optical density readings of BDNF protein levels were much greater (Fig. 13) in the regions of the brain where the blood-brain barrier is disrupted (IC and IH) compared to those where it remains intact (OC and OH). These preliminary findings suggest that intravenous injection of BDNF after FPI is effective in delivering it to the injured regions of the brain and that it may be neuroprotective. Further studies will be conducted in Year 3 of this project.

Proprietary data

7. Conclusions

1. BDNF protein levels were significantly increased in the bilateral hippocampus after FPI. These increases were observed 48 h after injury.

2. FPI did not produce significant alterations in BDNF protein in the cortex, or in trkB or ERK proteins in the cortex or hippocampus.

3. The FPI-induced increases in BDNF in the hippocampus did not lead to activation (phosphorylation) of ERK proteins in this same brain region.

4. Preliminary studies suggest that intravenous injection of BDNF 15 min to 24 h after FPI may be effectively targeting injured regions of the brain and that it may be neuroprotective, however additional studies need to be undertaken.

proprietary data

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Figure Legends

Figure 1. Summary diagram showing hypothetical relationship between traumatic brain injury (TBI), cell death, and BDNF/trkB signal transduction. Following experimental TBI, there is an increase in extracellular glutamate (Glu). Glu can raise intracellular calcium (Ca^{2+}) levels by binding to glutamate receptors (GluR). The increase in Ca^{2+} initiates a cascade of biochemical events, which can lead to cell death. The increase in glutamate may also be responsible for the up-regulation of BDNF mRNA following experimental TBI. BDNF mediates its effects by binding to its high-affinity receptor, trkB. BDNF/trkB interactions activate several intracellular kinases, including ERK. Activation of ERK involves phosphorylation, and this state can be detected with an antibody called anti-MAPK. BDNF/trkB signal transduction may promote cytoskeletal stability and the expression of calcium binding proteins, which may afford the cell neuroprotection following TBI.

Figure 2. BDNF protein levels in the injured cortex (IC) and opposite cortex (OC) were measured with ELISA techniques after FPI and compared to levels in sham (control) animals at corresponding times (n=4/group). Values are expressed as percentage of control level and represent the mean and SEM.

Figure 3. BDNF protein levels in the bilateral hippocampus were measured with ELISA techniques after FPI and compared to levels in sham (control) animals at corresponding times (n=4-6/group). Values are expressed as percentage of control level and represent the mean and SEM. *BDNF was significantly increased in the hippocampus at 48 h after FPI ($P < 0.003$).

Figure 4. NT-3 protein levels in the bilateral hippocampus were measured with ELISA techniques after FPI and compared to levels in sham (control) animals at corresponding times (n=4/group). Values are expressed as percentage of control level and represent the mean and SEM.

Figure 5. The truncated form of trkB (trkB-95) protein levels were measured in the injured cortex with Western blot techniques at various times after FPI (inj) and compared to levels in sham (sh) animals (n=4/group). Values are expressed as mean and SEM optical densities of the blots.

Figure 6. The full-length form of trkB (trkB-145) protein levels were measured in the injured cortex with Western blot techniques at various times after FPI (inj) and compared to levels in sham (sh) animals (n=4/group). Values are expressed as mean and SEM optical densities of the blots.

Figure 7. The truncated form of trkB (trkB-95) protein levels were measured in the bilateral hippocampus with Western blot techniques at various times after FPI (inj) and compared to levels in sham (sh) animals (n=4/group). Values are expressed as mean and SEM optical densities of the blots.

Figure 8. The full-length form of trkB (trkB-145) protein levels were measured in the bilateral hippocampus with Western blot techniques at various times after FPI (inj) and compared to levels in sham (sh) animals (n=4/group). Values are expressed as mean and SEM optical densities of the blots.

Figure 9. ERK protein levels were measured in the injured cortex with Western blot techniques and compared to levels in sham (sh) animals (n=4/group). Values are expressed as mean and SEM optical densities of the blots.

Figure 10. ERK protein levels were measured in the bilateral hippocampus with Western blot techniques and compared to levels in sham (sh) animals (n=4/group). Values are expressed as mean and SEM optical densities of the blots.

Figure 11. MAPK protein levels were measured in the bilateral hippocampus with Western blot techniques and compared to levels in sham (sh) animals (n=4/group). Values are expressed as mean and SEM optical densities of the blots.

Figure 12. The cortical lesion volume was calculated from tissue sections taken throughout the level of the lesion 7 d after FPI. Animals that received BDNF (10mg/kg, i.v.) either 15 min or 24 h after injury had a significantly smaller lesion volume than those that received saline injections ($P < 0.004$).

Figure 13. Intravenous injection of BDNF (10 mg/kg, i.v.) 15 min after a FPI resulted in increased levels of protein in the injured cortex (IC) and injured hippocampus (IH) compared to homotypic regions in the opposite cortex (OC) or opposite hippocampus (OH) in one animal. The increase was observed 2 hr after injury in regions that undergo opening of the blood-brain barrier following FPI.

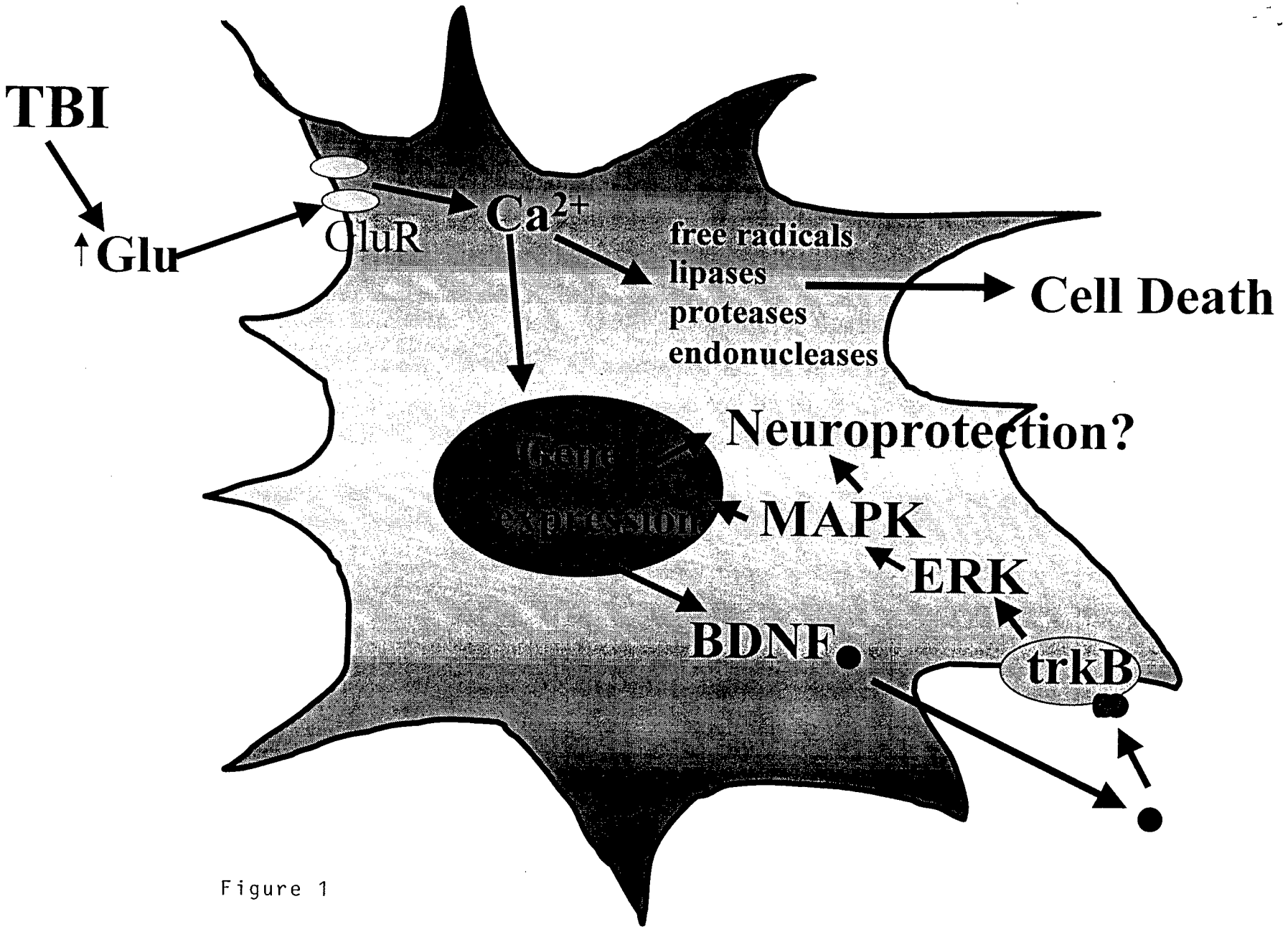
TBI
Glu
GluR
Ca^{2+}
free radicals
lipases
proteases
endonucleases
Cell Death
Neuroprotection?
MAPK
ERK
BDNF
trkB

Figure 1

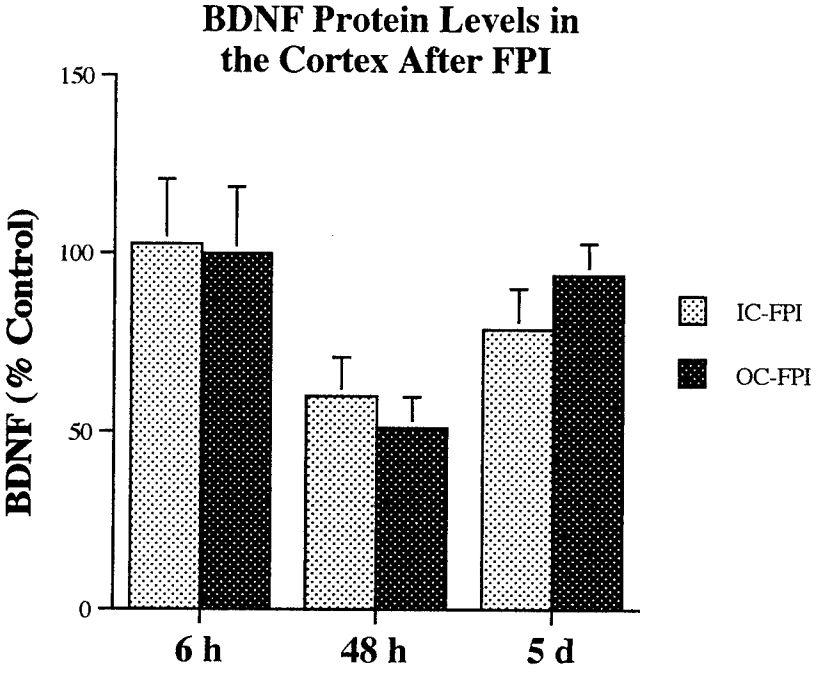
BDNF Protein Levels in
the Cortex After FPI
BDNF (% Control)
150
100
50
0
6 h
48 h
5 d
IC-FPI
OC-FPI

Figure 2

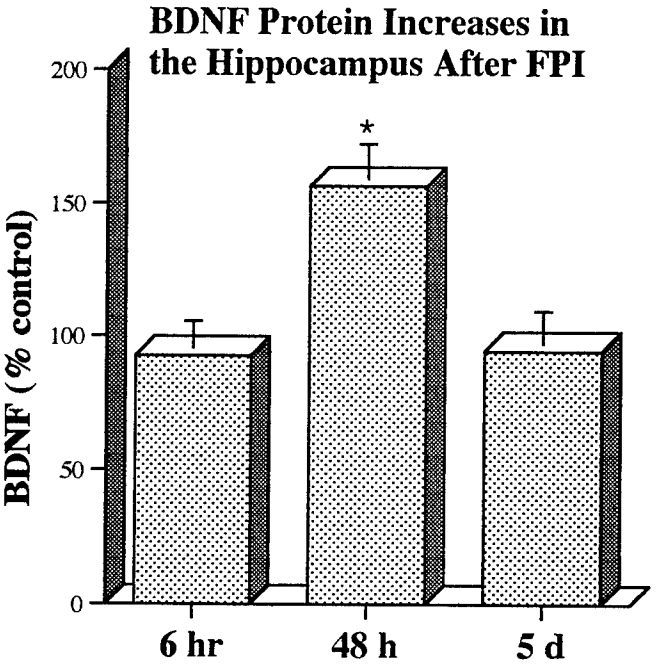
BDNF Protein Increases in
the Hippocampus After FPI
BDNF (% control)
200
150
100
50
0
*
6 hr
48 h
5 d

Figure 3

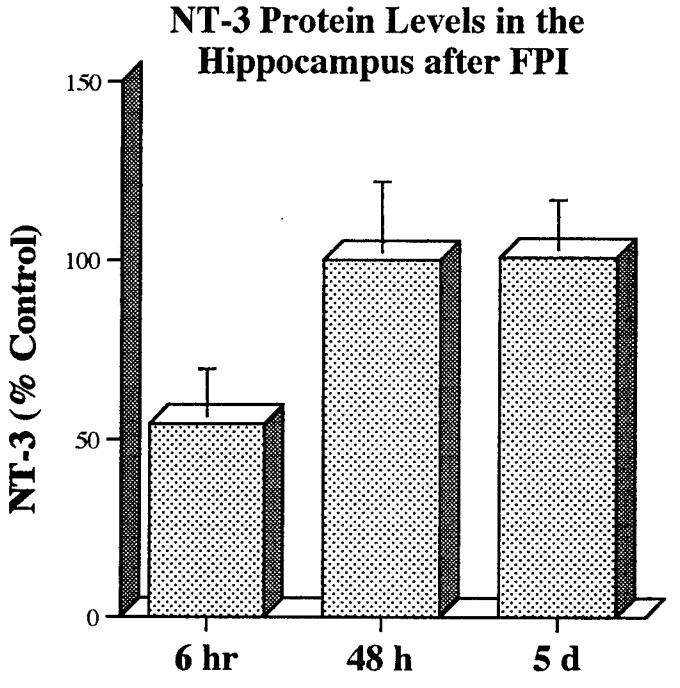
NT-3 Protein Levels in the Hippocampus after FPI
NT-3 (% Control)
150
100
50
0
6 hr
48 h
5 d

Figure 4

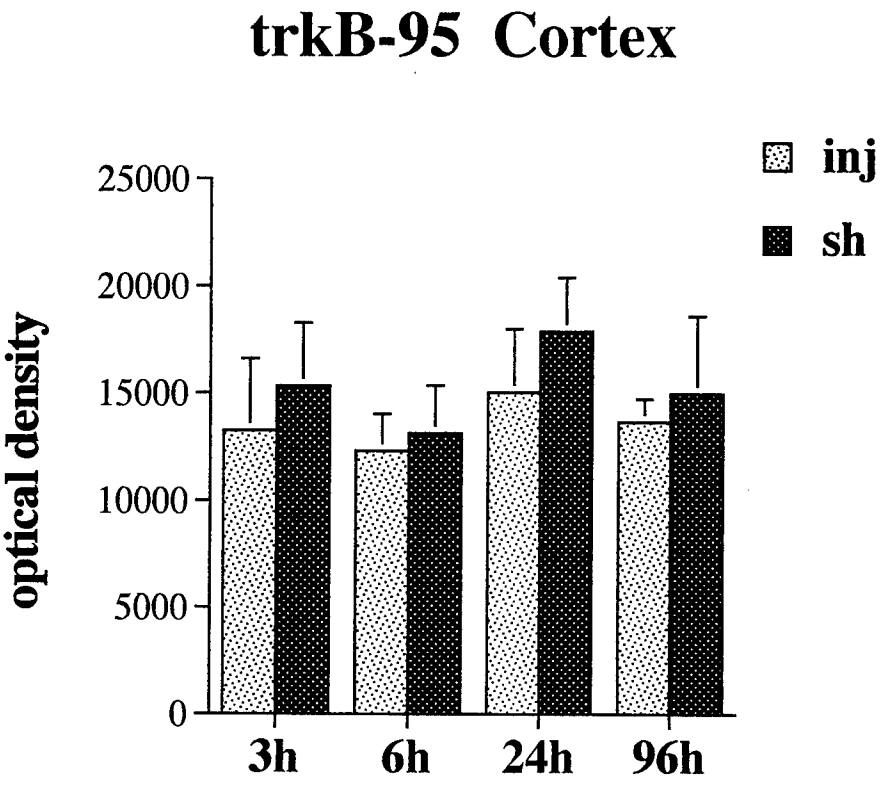
trkB-95 Cortex
inj
sh
optical density
25000
20000
15000
10000
5000
0
3h
6h
24h
96h

Figure 5

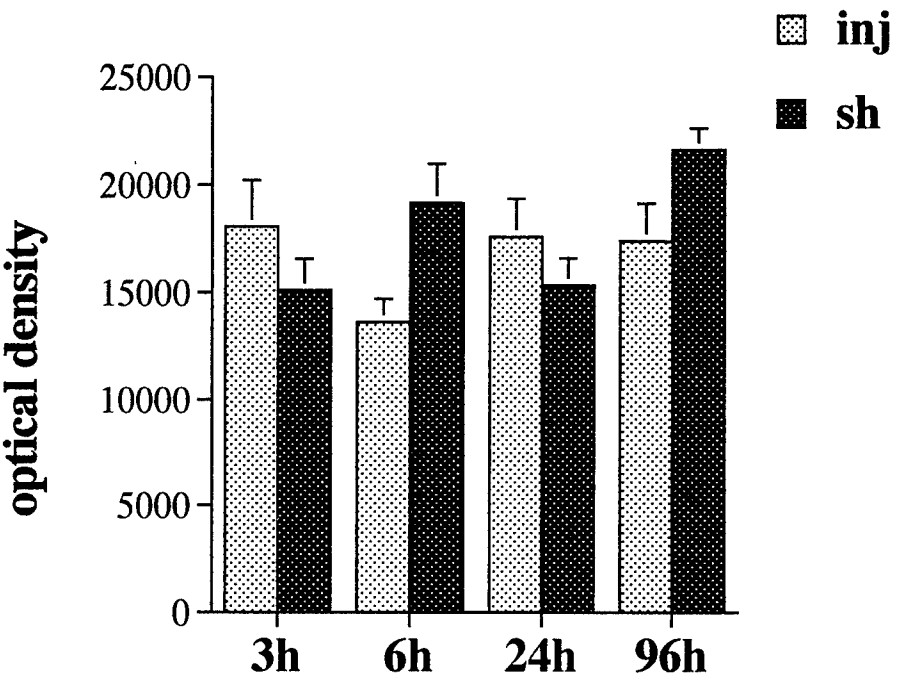
trkB-145 Cortex
inj
sh
optical density
25000
20000
15000
10000
5000
0
3h
6h
24h
96h

Figure6

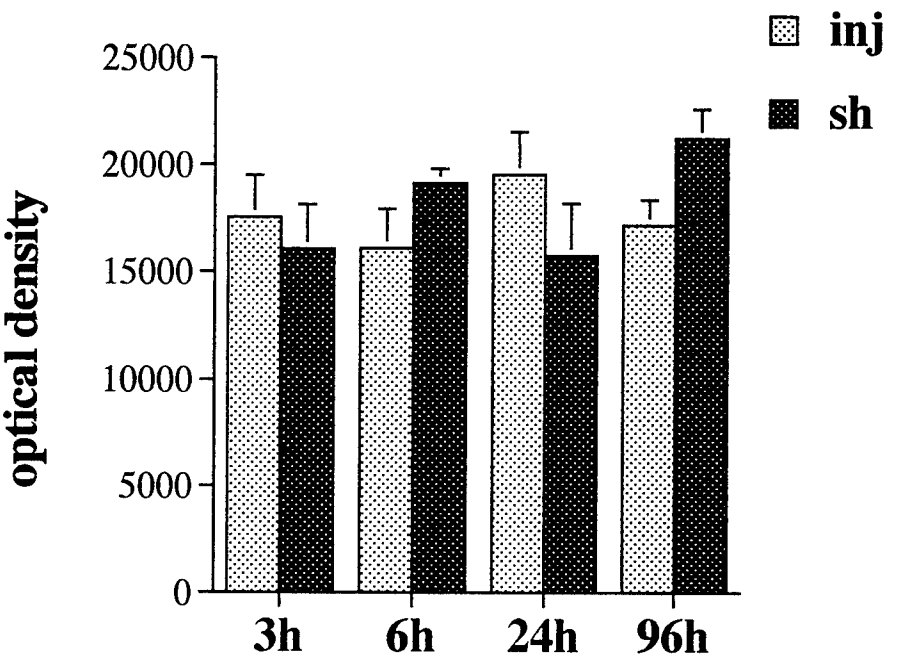
trkB-95 Hippocampus
inj
sh
optical density
25000
20000
15000
10000
5000
0
3h
6h
24h
96h

Figure 7

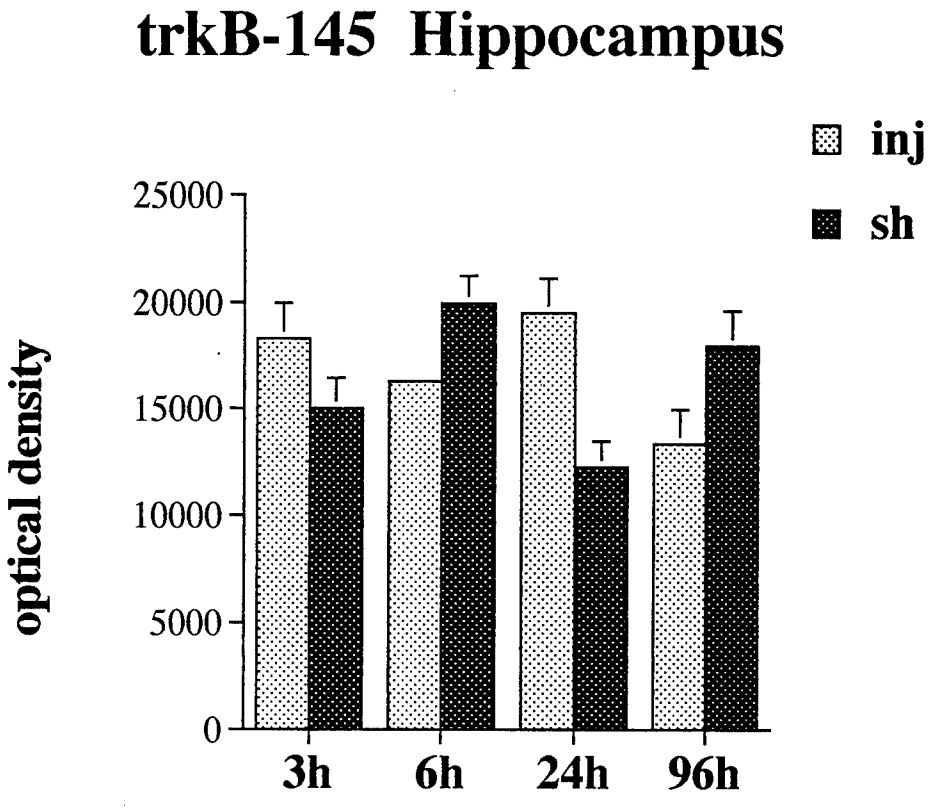
trkB-145 Hippocampus
inj
sh
optical density
25000
20000
15000
10000
5000
0
3h
6h
24h
96h

Figure 8

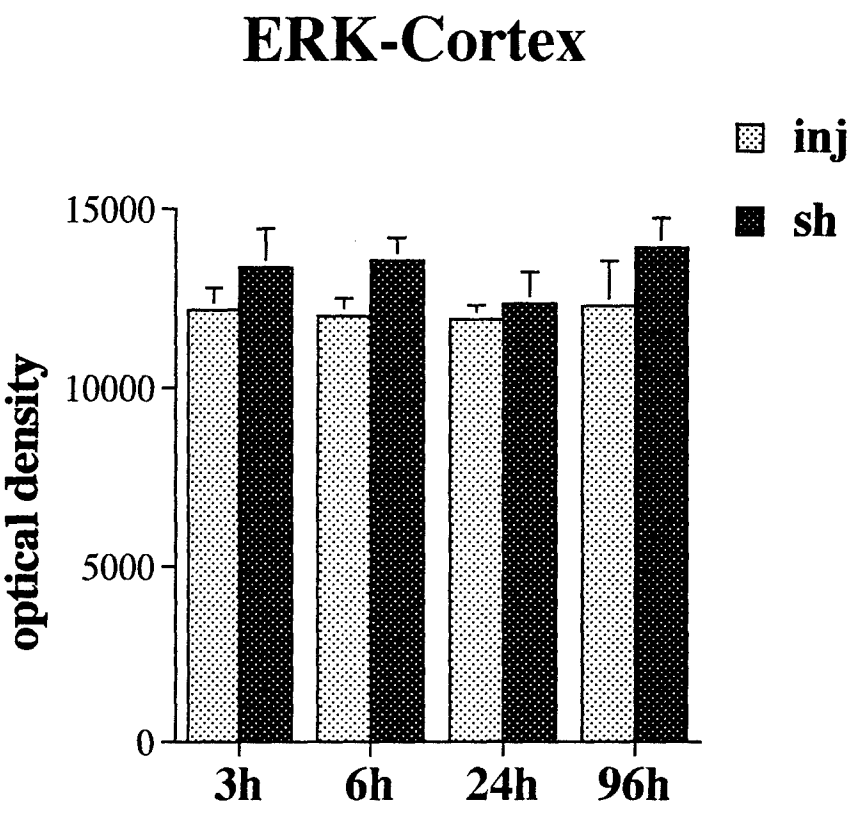
ERK-Cortex
inj
sh
optical density
15000
10000
5000
0
3h
6h
24h
96h

Figure 9

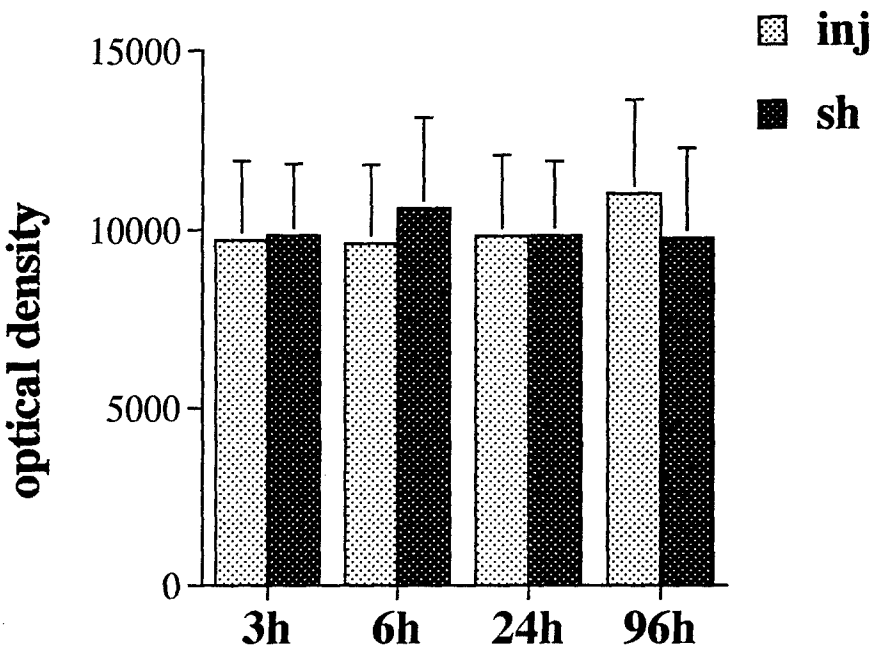
ERK-Hippocampus
inj
sh
optical density
15000
10000
5000
0
3h
6h
24h
96h

Figure 10

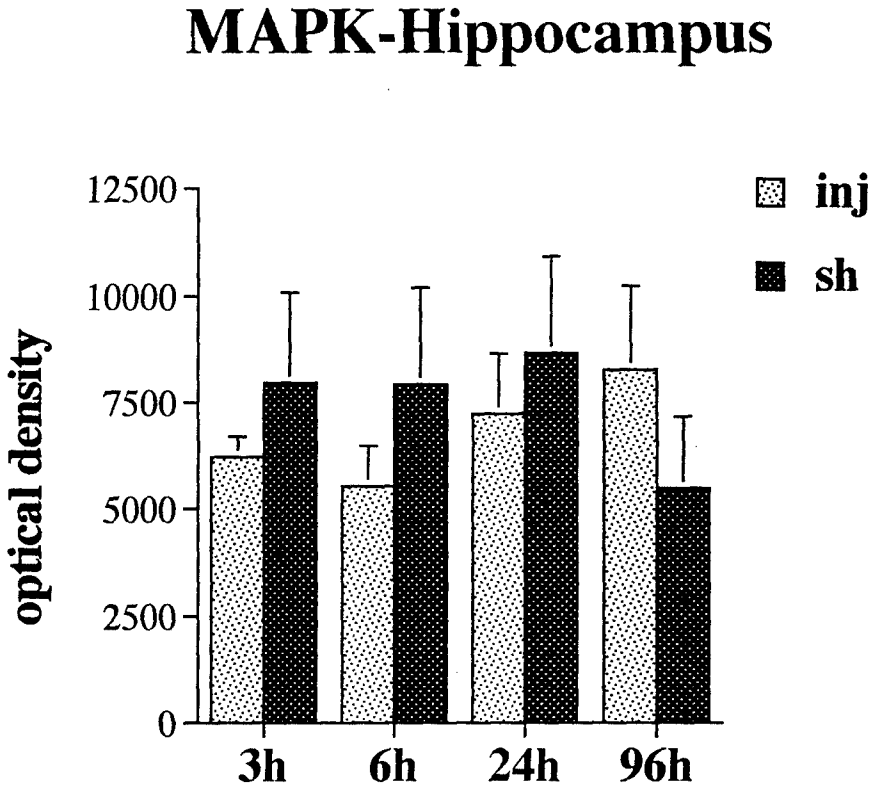
MAPK-Hippocampus
optical density
12500
10000
7500
5000
2500
0
3h
6h
24h
96h
inj
sh

Figure 11

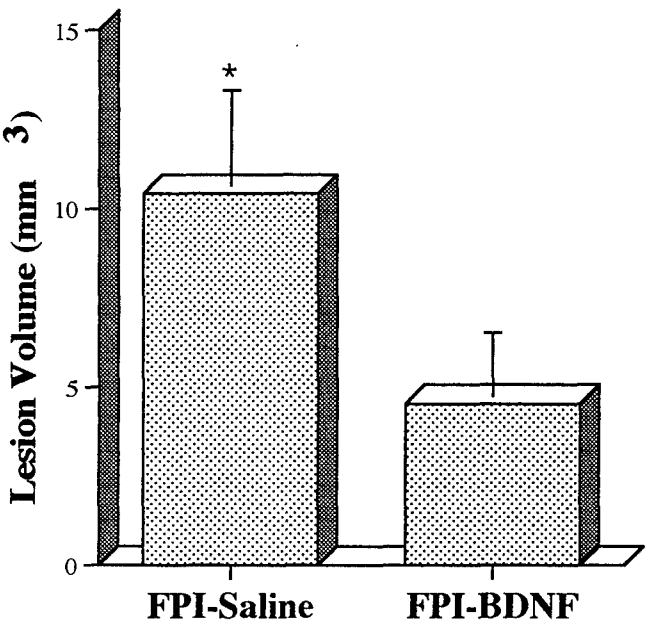

Figure 12 - Proprietary data

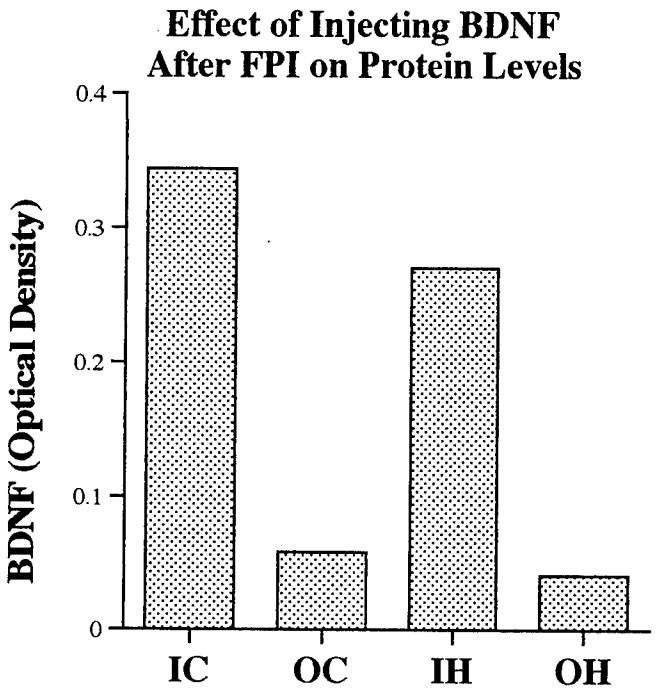

Figure 13 - Proprietary data

Appendix I

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Alterations in BDNF and trkB mRNA Levels in the Cerebral Cortex Following Experimental Brain Trauma in Rats

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Abstract

Recent studies have suggested that brain-derived neurotrophic factor (BNDF) and its receptor, trkB, may provide neuroprotection following injury to the central nervous system. Conversely, other studies have implicated BDNF as a contributing factor to neurodegenerative events that occur following injury. In order to further investigate the role of BDNF in neuroprotection, we subjected adult rats to a lateral fluid percussion (FP) injury of moderate severity (2.0 - 2.1 atm) or sham injury. After survival periods of 1, 3, 6, 24, or 72 h, the brains were processed for the in situ hybridization localization of BDNF and trkB mRNAs using ^{35}S-labeled cRNA probes. Hybridization levels were compared between injured and sham animals for regions of the cortex that were located within, adjacent to, and remote from the site of the cortical contusion. BDNF mRNA levels were significantly decreased in the injured cortex at 72 h, increased in adjacent cortical areas at 3 h, and increased bilaterally in the piriform cortex from 3 – 24 h post-FP injury. Expression of trkB mRNA was significantly decreased at all post-injury timepoints in the injured cortex and at 24 h in the adjacent cortex. These results demonstrate that following lateral FP injury, BDNF and trkB mRNA levels are decreased in cortical regions that contain degenerating neurons, generally unchanged in adjacent regions, and increased in remote areas. Thus, injury-induced decreases in the expression of BDNF and trkB may confer vulnerability to neurons within the cortical contusion.

Key Words: neurotrophic factors, traumatic brain injury, head injury

Introduction

Traumatic brain injury (TBI) is a major health problem, especially for adolescents and young adults. It accounts for 25% of injury-related deaths and is also a major cause of lifelong disability and impairment (Sosin et al., 1989). Although the neuropathology associated with TBI is variable, a cortical contusion is a frequently encountered form of neuronal injury (Adams, 1992). The lateral fluid percussion rodent model of experimental brain trauma effectively reproduces this feature of TBI, as a cortical contusion is the most pronounced form of neuropathology associated with this injury device (McIntosh et al., 1989; Hicks et al., 1996).

Previous studies have suggested that the neuronal damage following experimental TBI may initially be reversible (Cooper, 1985). It is believed that the impact and shear forces generated at the time of the injury cause primary neuronal damage, followed by a cascade of secondary excitotoxic biochemical events (Faden et al., 1989; Katayama et al., 1990). Identifying pharmacologic agents to block these secondary neurodegenerative events has been a primary focus of TBI research. To this end, administration of the trophic factor basic fibroblast growth factor has been reported to result in a significant decrease in the cortical contusion volume following FP injury in rats (Dietrich et al., 1996).

Another potentially neuroprotective agent for TBI is brain-derived neurotrophic factor (BDNF). Numerous studies have demonstrated that administration of BNDF can protect cells from a variety of central nervous system insults both in vitro (Skaper et al., 1993; Cheng and Mattson, 1994; Hayes et al., 1995; Mattson et al., 1995; Kubo et al., 1995; Nakao et al., 1995) and in vivo (Kindy, 1993; Beck et al., 1994; Mansour-Robey et

al., 1994; Yurek et al., 1996; Cheng et al., 1997; Schabitz et al., 1997; Klocker et al., 1998; Hagg, 1998). However, there is also evidence to suggest that BDNF potentiates neuronal injury in vitro (Koh et al., 1995) and in vivo (Rudge et al., 1998).

BDNF mediates its effects through interaction with a tyrosine kinase receptor, trkB (Barbacid, 1994; Lindsay et al., 1994). Both BDNF and trkB mRNA levels increase significantly in the hippocampus following lateral FP (Hicks et al., 1997; 1998) and cortical impact injury (Yang et al., 1996). In the present study, we investigated time-dependent alterations in expression of BNDF and trkB mRNAs in regions of the cortex that undergo varying degrees of neuronal degeneration following a lateral FP injury.

Methods

Surgery and Fluid Percussion Injury

Male Sprague-Dawley rats (325-350 g) were injected with atropine (0.15 ml i.m.) 10 min prior to being anesthetized with sodium pentobarbital (60 mg/kg i.p.), and placed in a stereotaxic frame. After reflecting the scalp and temporal muscles, the animals were given a 5 mm craniotomy with a hand-held Michele trephine over the left parieto-occipital cortex. The craniotomy was centered between bregma and lambda, and was lateral to the sagittal suture. A hollow Luer-Lok fitting was fixed rigidly with dental cement to the craniotomy. Experimental brain injury of moderate severity (2.0 - 2.1 atm) was induced in the anesthetized animals (n=20), using the lateral FP brain injury model. This model is well-characterized and has been previously described in detail (McIntosh et al., 1989). Following FP injury, rats were euthanatized at 1, 3, 6, 24, or 72 h (n=4/survival period), in order to assess the acute response of BDNF and trkB mRNAs to

the injury. Additional animals (n=15; 3/survival period) underwent anesthesia and surgery but were not injured (sham injury).

After the appropriate survival times, the rats were deeply anesthetized with an overdose of sodium pentobarbital and decapitated. Brains were rapidly removed and frozen over dry ice. Tissue sections through the hippocampus were cut in the coronal plane at 10 μm in a cryostat, thaw-mounted onto Superfrost Plus (Fisher Scientific) glass slides, and stored at -20°C until processing for hybridization. Adjacent sections throughout the cerebral cortex of animals from the various injury and sham groups were processed for the in situ hybridization localization of mRNAs for BDNF and trkB as previously described [Gall et al., 1992; Seroogy et al., 1994; Numan and Seroogy, 1997; Seroogy and Herman, 1997]. The cRNA probes were prepared by in vitro transcription from linearized cDNA constructs with the appropriate RNA polymerase in the presence of ^{35}S-UTP. The 540-base BDNF cRNA (plasmid kindly provided by J. Lauterborn and C. Gall, Univeristy of California, Irvine) includes 384 bases complementary to the rat BDNF mRNA coding region [Isackson et al., 1991; Gall et al., 1992]. The cDNA construct for trkB (plasmid kindly provided by D. McKinnon, State University of New York at Stony Brook) resulted in an antisense RNA transcript that was 196 bases in length. The trkB cRNA probe detects the kinase-specific, full-length catalytic form of the receptor mRNA [Middlemas et al., 1991; Goodness et al., 1997]. Hybridization was conducted at 60°C for 18-24 h with the ^{35}S-labeled cRNAs at a concentration of 1 x 10^6 cpm/50μl/slide. Following post-hybridization washes and ribonuclease treatment, the sections were air-dried and exposed to β-Max Hyperfilm (Amersham) for 14-18 days at room temperature for generation of film autoradiograms. After autoradiographic film

development, the sections were dipped in NTB2 nuclear track emulsion (Kodak; 1:1 in H_2O), air-dried, and exposed in light-tight boxes at 4°C for 4-6 weeks. After autoradiographic development of the emulsion, the sections were counterstained with cresyl violet, coverslipped in D.P.X. mounting medium (Fluka), and analyzed with a Nikon Optiphot-2 microscope equipped with brightfield and darkfield optics. Cells were considered labeled if the density of reduced silver grains overlying the perikarya was at least tenfold greater than background. Control sections that had been treated with ribonuclease A (45°C for 30 min) before hybridization or processed for hybridization with appropriate sense-strand riboprobes were devoid of specific labeling.

Film autoradiograms were analyzed with Image 1.60 software (NIH) to measure the density of hybridization for BDNF and trkB mRNAs in the following cortical regions: injured cortex (IC), superficial and deep layers of the adjacent cortex (AC) and opposite (homotypic) cortex (OC), and the bilateral piriform cortex (PC) (see Fig. 1). Background optical density (O.D.) measurements were taken in adjacent white matter of the corpus callosum and subtracted from the O.D. measurements in the cortical regions in order to obtain corrected O.D. measurements. Hybridization levels observed after lateral FP injury were compared to those found after sham injury with equivalent survival periods. At least three sections were analyzed per animal. All measurements are expressed as the mean values plus or minus the standard error of the mean (SEM). The effects of treatment (injury vs. sham), survival time, and their interaction effects were analyzed with a two-way analysis of variance (ANOVA) for each cortical region. BDNF and trkB mRNA levels did not differ by side for the PC. Therefore, the hybridization data from

the right and left sides were combined. Bonferonni post-hoc analyses were used for pairwise comparisons with a significance set at $P < 0.05$.

Results

Hybridization for BDNF and trkB mRNAs in the cortex in the control (sham injury) animals (Figs. 2A and 4A), was similar to previous reports described for normal, uninjured rats (Ernfors et al., 1990; Fryer et al., 1996). Following FP injury, BDNF mRNA levels in the IC did not differ from sham levels until 72 h, when they were significantly decreased ($P < 0.01$) (Figs. 2F and 3A). This is in contrast to the superficial layers of the AC, where BDNF mRNA was significantly increased at 3 h post-injury ($P < 0.02$) (Figs. 2C and 3B), and to the PC, where levels were significantly increased bilaterally ($P < 0.02$) at 3, 6, and 24 h in the FP injured-animals (Figs. 2C,D,E and 3C), compared to shams. In the deep layers of the AC and in all layers of the OC (Fig. 2), densitometric measurements revealed no time-dependent changes in BDNF mRNA after FP injury (quantitative data not shown).

The response of cortical trkB mRNA expression to FP injury was generally quite different from that of BDNF mRNA. No cortical regions for any of the survival periods following FP injury demonstrated an increase in hybridization for trkB mRNA. Rather than an increase, expression of trkB mRNA in the IC was significantly decreased ($P < 0.001$) at all times following the FP injury compared to sham animals (Figs. 4 and 5A). There was also a significant decrease ($P < 0.03$) of trkB mRNA levels in the superficial layers of the AC at 24 h post-FP injury (Figs. 4E, 5B). In the deep layers of the AC, all

layers of the OC, and bilaterally in the PC, no time-dependent alterations in trkB mRNA were observed after FP injury (Fig. 4; quantitative data not shown).

Discussion

The purpose of this study was to characterize the response of BDNF and trkB mRNAs to lateral FP brain injury in cortical regions that undergo varying degrees of neurodegeneration. To this end, we evaluated time-dependent changes in BDNF and trkB mRNA levels in cortical tissue within, adjacent to, and remote from the contusion site. The hybridization levels for BDNF mRNA varied by region, with a persistent increase in the bilateral PC, a transient increase in the superficial layers of the AC, no change in the deep layers of the AC, no change in the OC, and a delayed decrease in the IC. These cortical alterations in BDNF mRNA are in general agreement with those observed in a different model of experimental brain trauma, the cortical impact injury (Yang et al., 1996). The hybridization levels for trkB mRNA did not change in the cortex following FP injury, except for an immediate and persistent decrease in the IC, and a transient decrease in the superficial layers of the AC.

The most pronounced increases in BDNF mRNA hybridization levels were observed in the bilateral PC at 3, 6, and 24 h post-FP injury. Conversely, trkB mRNA levels remained unchanged in these cortical regions. Similar observations have been reported following seizures, with significant increases in BDNF mRNA, and no change in trkB mRNA in the PC between 1 and 24 h post-kindling (Ernfors et al., 1991; Kokaia et al., 1996a). However, other studies have not only observed increases in BDNF mRNA in the PC, but also trkB mRNA 3-4 h following seizures (Mudo et al., 1993; 1996).

The PC has been described as a selectively vulnerable region for seizures (Tanaka et al., 1996). The possibility that the increases in BNDF mRNA in the PC following FP injury are attributable to seizures cannot be completely eliminated in the present study. However, evidence against this possibility include the absence of abnormal behavior or overt seizure activity in any of the animals in this study, or in a previous study of moderate FP injury (Lowenstein et al., 1992), and a lack of bilateral neurochemical changes that would be expected with seizures (Padmaperuma et al., 1996; Prasad et al., 1994). Furthermore, increases in BDNF mRNA in the neocortex following FP injury differ from those observed following seizures. FP injury-induced changes are restricted to the superficial layers of the neocortex, whereas seizure-induced changes are observed in all neocortical layers (Gall, 1993).

Another possible explanation for the robust increase of BDNF mRNA in the PC, a region that is remote from the FP injury and associated with a role in memory and learning (Litaudon et al., 1997), may be its relatively high density of glutamate receptors (Sato et al., 1995). The hippocampus, another region with high levels of glutamate receptors (Sato et al., 1995), also undergoes marked increases in BDNF expression following FP injury (Hicks et al., 1997). Previous studies have demonstrated that basal levels of BDNF are regulated by *N*-methyl-D-aspartate (NMDA) receptor activation, whereas alterations in response to acute injuries are regulated by non-NMDA receptors (Lindholm et al., 1994; Lindvall et al., 1992; Wetmore et al., 1994). Glutamate increases after FP injury (Faden et al., 1989; Katayama et al., 1990) and may thus mediate the increases in BDNF mRNA expression in the PC.

The results of this study also suggest that up-regulation of BDNF/trkB mRNA within the IC does not contribute to neuronal degeneration as hybridization remained at or below control levels for all time points. These findings are consistent with those observed following ischemic injury (Kokaia et al., 1996b). However, it does not eliminate the possibility that anterograde transport of BDNF from the AC may contribute to degenerative events. Anterograde transport of BDNF in mossy fibers of the dentate gyrus following kainic acid injections is believed to exacerbate the neuronal degeneration in the CA3 pyramidal cells of the hippocampus (Rudge et al., 1998). This appears unlikely following FP injury, though, because whereas cortico-cortical connections are prevalent between neurons in adjacent cortical regions in layers II and III (Kolb, 1990), neuronal degeneration is observed fairly uniformly throughout all layers (Hicks et al., 1996).

However, the cortical afferent projections from the IC may be related to the transient increase in BDNF mRNA that is observed at 3 h post-injury in the AC, because the increase was localized to cells in the superficial but not the deep layers of this region. Alternatively, because non-NMDA receptors are more prevalent in the superficial than deep layers of the neocortex (Huntley et al., 1994), the differential response between layers may be attributable to post-FP injury elevations in glutamate. Similar increases in BDNF hybridization were not observed in the OC. Thus, while adjacent and opposite cortical regions have been observed to undergo cortical reorganization following various injuries and are hypothesized to play a role in recovery of function (Barneoud et al., 1991; Castro-Almancos and Borrel, 1995; Dietrich et al., 1987; Dunn-Meynell and Levin,

1995), we did not observe sustained alterations in BDNF or trkB mRNA in either of these cortical regions, at least during the initial (72 h) period following FP injury.

In summary, lateral FP injury produces alterations in BDNF and trkB mRNA levels in several regions of the cerebral cortex. The largest increase in BDNF mRNA following FP injury was observed in the bilateral PC, regions relatively remote from the impact and injury sites. The functional significance of the increase in the bilateral PC remains to be determined. The IC, the site of the contusion, demonstrates normal or below normal levels for both BDNF and trkB mRNA throughout the acute period following injury, suggesting that up-regulation and enhanced activation of BDNF/trkB signal transduction pathways are not contributing to the neuropathology. However, it is possible that the injury-induced loss in expression of BDNF and trkB may confer vulnerability to neurons within the cortical contusion. Other neocortical regions either showed no significant alterations, or changes of very short duration, suggesting that injury-induced regulation of BDNF/trkB pathways is somewhat unlikely to have a significant influence on neural recovery, at least during the acute periods following FP injury. Future studies are needed to examine the effects of manipulating BDNF/trkB signal transduction activity on neuroprotection following lateral FP brain injury.

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at the University of Kentucky and the Guide for Care and Use of Laboratory Animals from the US Department of Health and Human Services.

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Figure Legends

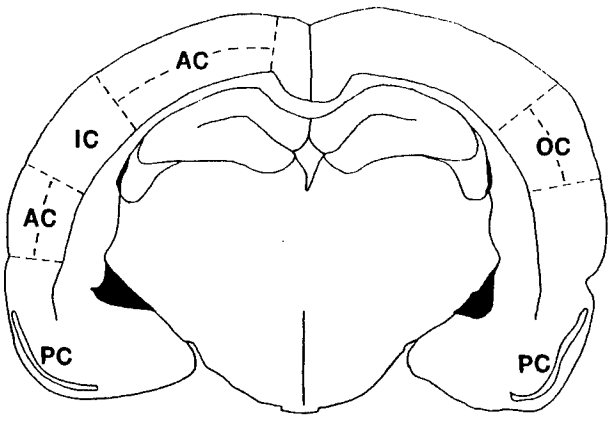
Fig. 1. Schematic representation of cortical regions analyzed for optical density measurements of hybridization for BDNF and trkB mRNA; IC, the cortical region that comprises the lesion site following lateral FP injury; AC, the adjacent neocortical regions that surround the injury site; OC, the cortical region on the opposite side of the brain that is comparable in size and location to the IC; and PC, the bilateral piriform cortices. The AC and OC were subdivided into superficial and deep layers.

Fig. 2. Prints of autoradiograms showing expression of BDNF mRNA in coronal sections from a rat 3 h after a sham injury (A), 3 h after a lateral FP injury (B), 72 h after a sham treatment (C), and 72 h after a FP injury (D). Note the up-regulation of BDNF 3 h after a FP injury in the bilateral piriform cortices (arrowheads, B) and in the superficial layers of the adjacent cortex (arrows, B), and the decrease in BDNF mRNA in the injured cortex at 72 h post-injury (arrowheads, D). Scale bar = 1,000 μm.

Fig. 3. Graphs showing corrected optical density (O.D.) measurements of hybridization for BDNF mRNA in the injured cortex (A), superficial layers of the adjacent cortex (B), and bilateral piriform cortex (C) over time following lateral FP brain injury. Note the significant decrease in BDNF mRNA expression in the injured cortex at 72 h following injury (A), and the significant increase in the superficial layers of the adjacent cortex at 3 h post-injury (B), and in the bilateral piriform cortex at 3, 6, and 24 h following injury (C) compared to the sham injury groups ($*P < 0.02$).

Fig. 4. Film autoradiograms showing expression of trkB mRNA in coronal sections from a rat 24 h after a sham injury (A), and 24 h after a lateral FP injury (B). Note the decreased hybridization for trkB mRNA in the injured cortex (arrows) and in the superficial layers of the adjacent cortex (arrowheads) following injury (B). Scale bar = 1,000 μm.

Fig. 5. Graphs showing corrected optical density (O.D.) measurements of hybridization for trkB mRNA in the injured cortex (A) and superficial layers of the adjacent cortex (B) over time following lateral FP brain injury. Note the significant decrease in BDNF mRNA expression in the injured cortex at all time points following injury (A), and at 24 h in the superficial layers of the adjacent cortex (B) compared to the sham injury groups ($*P < 0.03$).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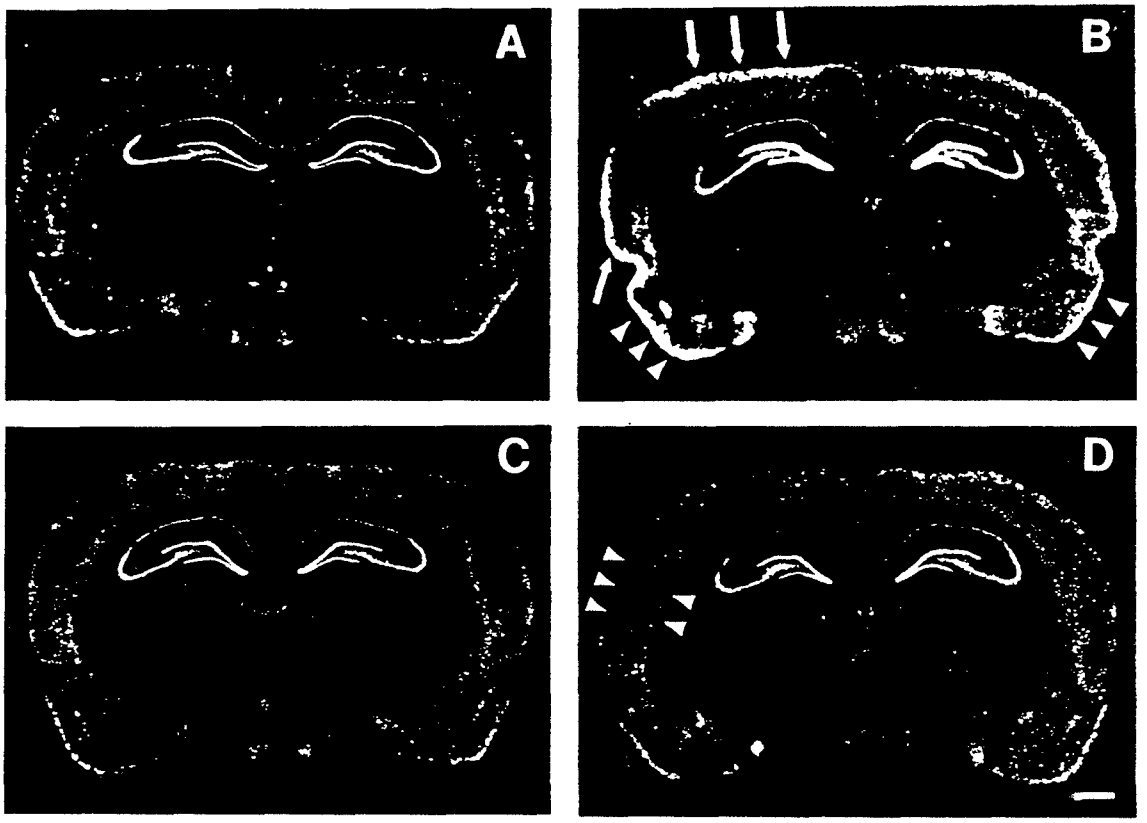
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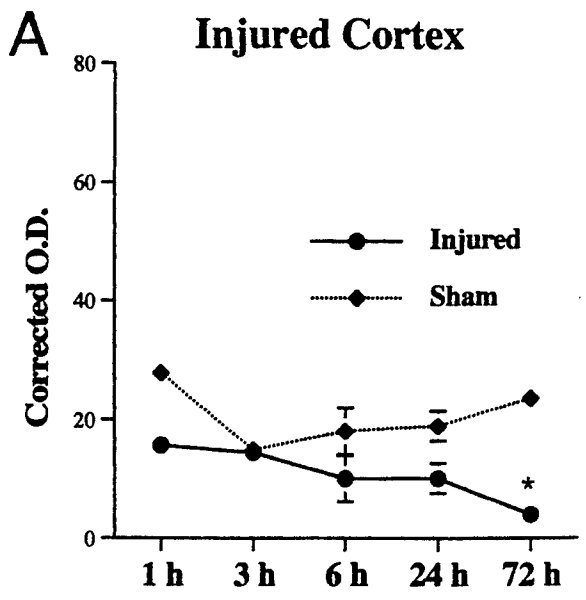
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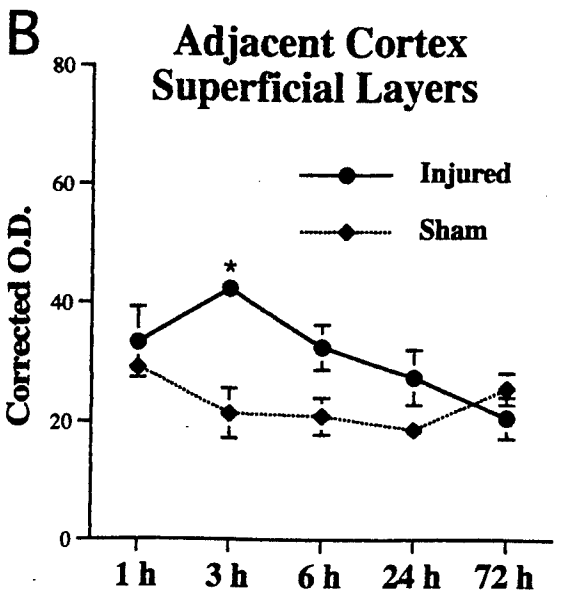
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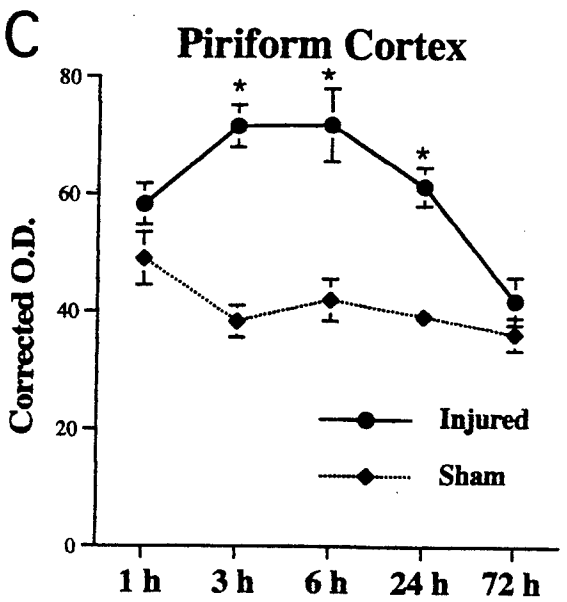
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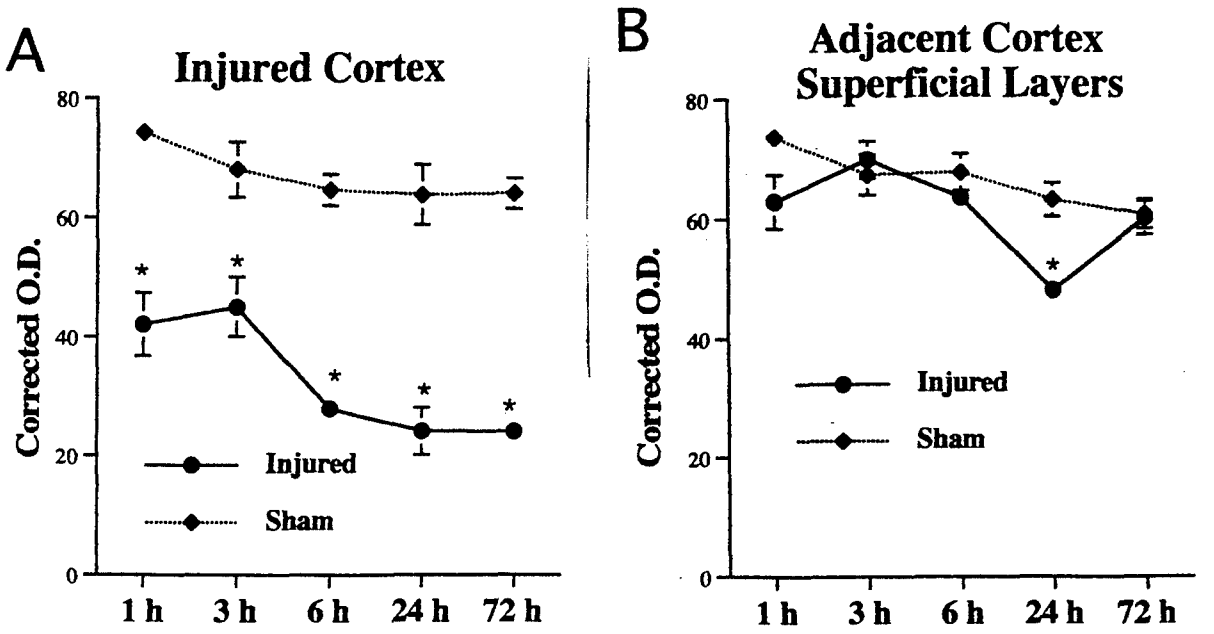
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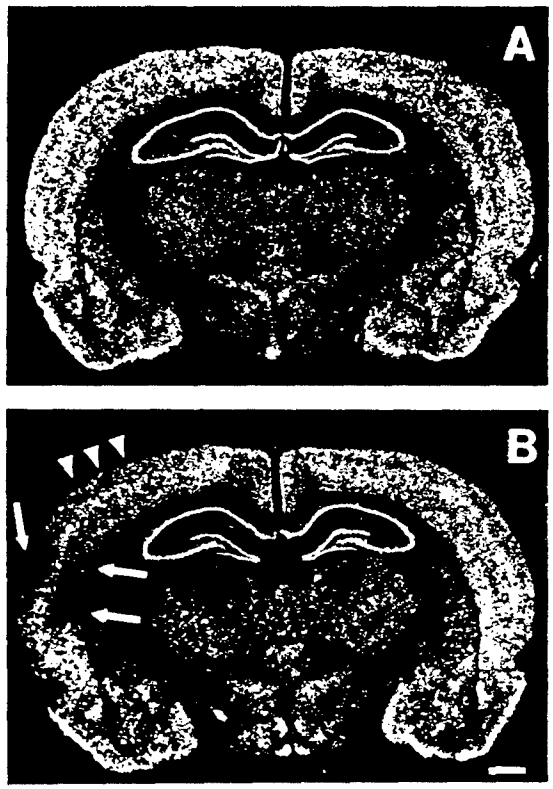
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Appendix II

In Preparation for

Submission to Exp Neurol

MILD EXPERIMENTAL BRAIN INJURY ALTERS THE EXPRESSION OF NEUROTROPHIC FACTORS IN THE HIPPOCAMPUS

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Abstract

The molecular events responsible for impairments in cognition following mild traumatic brain injury are poorly understood. Neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), have been identified as having a role in learning and memory. We have previously demonstrated that following experimental brain trauma of moderate severity, BDNF and its receptor, trkB, mRNA levels are increased in the bilateral hippocampus for several hours. In the present study, we used in situ hybridization to compare BDNF, trkB, neurotrophin-3 (NT-3), and trkC mRNA expression in rat hippocampus at 3 or 6 h after a lateral fluid percussion brain injury (FP) of mild severity (1.0 atm) to sham injured controls at equivalent time points. Significant alterations in hybridization levels for BDNF, trkB, and NT-3 mRNA were present in the hippocampus, but only on the side ipsilateral to the FP injury. These findings suggest that even a mild traumatic brain injury may alter neurotrophin signal transduction pathways in the hippocampus. Such alterations may have important implications for neural plasticity and recovery of function.

Keywords: BDNF, NT-3, trkB, trkC, hippocampus, traumatic brain injury

Introduction

Clinically, the vast majority of head injuries (75-90%) are classified as mild (Langfitt and Gennarelli, 1982; Kraus and Nourjah, 1988), because post-traumatic amnesia is present for less than 24 hours (Jennett and MacMillan, 1981). Despite the rather benign acute symptoms, 50% of the individuals who sustain a mild head injury demonstrate residual impairments 1 year later (Watson et al., 1995). These impairments include cognitive deficits (Parker and Rosenblum, 1996), emotional disturbances (Parker, 1996), and abnormal EEG recordings (Watson et al., 1995). Relatively little is known about the neuropathological consequences of mild head injury and how they might contribute to these functional deficits.

Neurotrophic factors (NTFs) are a family of structurally related polypeptides that have been shown to play a critical role during neuronal development and appear to mediate a protective response in mature animals (Hefti et al., 1989; Mattson and Scheff, 1994). Members of the NTF family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin 4/5 (NT-4/5), and neurotrophin 6 (NT-6) (Thoenen, 1991). NTFs are believed to promote cell survival through their interactions with a common low-affinity receptor, called p75 (Rabizadeh et al., 1993; Carter et al., 1996). NTFs also interact with specific high-affinity tyrosine kinase (trk) receptors, which mediate different intracellular signal transduction pathways (Meakin and Shooter, 1992; Barbacid, 1994).

Numerous investigations are currently underway to elaborate the specific actions of the various NTFs and how they may promote survival or neural recovery after brain injury. In brief, NGF/trkA interactions are believed to be important for cell survival

during development and following injury (for review, see Mattson and Scheff, 1994), and to promote sprouting and regeneration of connections of cholinergic neurons (Dekker et al., 1994; Tuszynski et al., 1990). In an animal model of TBI, infusion of NGF into the lesion improved cognitive function without overt effects on neuropathology (Sinson et al, 1995). BDNF is the most abundantly expressed NTF in the mature central nervous system (Hofer et al., 1990) and supports the survival of many types of neurons (Lindsay, 1993). BDNF/trkB interactions appear to be neuroprotective following various forms of brain injury (Beck et al., 1994; Cheng & Mattson 1994; Hayes et al., 1995; Kindy 1993; Kubo et al., 1995; Schabitz et al., 1997; Skaper et al., 1993), although this is controversial as a few studies have implicated it as a contributing factor to neuronal degeneration (Koh et al., 1995; Rudge et al., 1998). BDNF also appears to be important for learning and memory, including a role in long-term potentiation (Dragunow et al., 1997; Figurov et al., 1996; Korte et al., 1996), dendridogenesis (McAllister et al., ???) and activity-dependent neuroplasticity (Gall 1992; Rocamora et al., 1996). NT-3/trkC signal transduction has also been found to have neuroprotective effects in some models of neuronal injury (Hagg 1997; Lindsay 1993). Less is known about the other NTF/trk interactions, but they may also have a role in neuroprotection following various CNS disorders (Mattson & Scheff, 1994; Lindsay 1993).

The expression of BDNF, trkB, and NT-3 in the hippocampus is altered following experimental traumatic brain injury (FPI) of *moderate* severity (Hicks et al., 1997b, 1998; Yang et al., 1996). Much less is known about alterations in NTF expression following an experimental brain injury of *mild* severity. For these reasons, we examined mRNA levels of BDNF, NT-3, trkB, and trkC following mild experimental brain injury in the

hippocampus. The hippocampus was of particular interest because of its high level of NTF expression in adult animals, its role in memory and learning, and its selective vulnerability following many types of central nervous system insults.

Material and Methods

Surgical Procedures

Mild lateral FPI (1.0 atm) or sham-injury was produced in male Sprague-Dawley rats (n=14, 300-350g) as previously described (McIntosh et al., 1989; Hicks et al., 1993). The rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.) 10 minutes after receiving 0.15 ml of atropine (0.4 mg/ml i.m.). The head was rigidly fixed in a stereotaxic frame while the scalp and temporal muscles were reflected, and a 5.0 mm craniotomy was made with a hand-held trephine over the parieto-occipital cortex. The craniotomy was centered between bregma and lambda, 3 mm lateral to the sagittal suture. A Luer-Loc hub was fixed to the craniotomy with dental acrylic and filled with sterile saline. A fluid percussion device (Dept. of Biomedical Engineering, Medical College of Virginia) was used to induce a lateral FPI of mild severity in the anesthetized animals. Following FPI, rats were allowed to survive 3 or 6 h before euthanasia. These time points were selected because following moderate FPI, peak alterations in BDNF and trkB gene expression occurred at 3 and 6 hr post-injury (Hicks et al., 1997b, 1998).

Tissue Processing

Following deep anesthesia with an overdose of sodium pentobarbital, the animals were decapitated, the brain rapidly removed and frozen over dry ice. Coronal brain

sections (10 μm) were cut in a cryostat, thaw-mounted onto Superfrost Plus (Curtin Matheson Scientific) glass slides, and stored at –20°C until processing for hybridization. **Ask Kim to finish methods for tissue processing.**

Quantitative Image Analysis

Film autoradiograms were digitized and analyzed with image processing software (Image 1.60, NIH). At least 4 sections were analyzed per animal to compare the density of hybridization for BDNF, NT-3, trkB, and trkC in the hippocampus between the FPI and sham groups. Optical densities (O.D.) of BDNF, trkB, and trkC hybridization were measured in the stratum granulosum of the dentate gyrus, and in the strata pyramidale of CA1 and CA3 for the left (ipsilateral to the impact site) and right (contralateral to the impact site) sides of the brain for the FPI group. Right and left side data for the sham animals was combined. NT-3 hybridization was only measured in the dentate gyrus, because its expression is localized to this region and CA2, but not the other subfields (Ernfors et al., 1990). Background O.D. measurements were taken in adjacent white matter of the corpus callosum and subtracted from the O.D. measurements in the hippocampus in order to obtain corrected values. All measurements are expressed as the mean values plus or minus the standard error of the mean (S.E.M.). The effects of group (FPI - ipsilateral, FPI - contralateral, and sham - bilateral), survival time (3 h and 6 h) and their interaction effects were analyzed for each hippocampal subfield with a two-way analysis of variance (ANOVA). Bonferonni post-hoc analyses were used for pairwise comparisons with a significance set at $P < 0.05$.

Results

Hybridization in the sham animals for BDNF, NT-3, trkB and trkC mRNAs was similar to previous reports in normal, uninjured rats (Altar et al., 1994; Fryer et al., 1996; Merlio et al., 1992; Ernfors et al., 1990; Isackson et al., 1991).

BDNF mRNA. Three or six hours following FPI, increases in BDNF mRNA were evident in the dentate gyrus and CA3 regions of the hippocampus on the ipsilateral side of the brain compared to the contralateral side or the sham animals (Fig. 1A, E). Statistical analysis revealed no differences between the 3 and 6 h survival times, but there were significant differences across groups. O.D. measurements of BDNF mRNA in the ipsilateral dentate gyrus were significantly greater than on the contralateral side or in sham animals ($P < 0.001$). Similarly, CA3 O.D. measurements were greater on the ipsilateral than on the coontralateral side after FPI, and greater than sham animals ($P < 0.01$). Small, but significant increases were also observed in the CA1 region on the ipsilateral side of the brain compared to sham animals ($P < 0.05$) (Fig. 2).

trkB mRNA. The FPI induced marked increases in trkB hybridization O.D. in the dentate gyrus on the ipsilateral side of the brain compared to the contralateral side or sham animals (Fig. 1B, F). Alterations were not observed in the CA1 and CA3 regions (Fig. 1B, F). Statistical analysis confirmed that there was a significant difference in trkB mRNA levels in the dentate gyrus between groups ($P < 0.001$), but not between survival times (Fig. 3).

NT-3 mRNA. Hybridization for NT-3 mRNA was visibly decreased following FPI in the ipsilateral dentate gyrus compared to the contralateral side or sham animals (Fig. 1C, G). Statistical analysis revealed that there were time and group differences, and a

significant interaction between them. 3 h post-FPI, NT-3 mRNA levels were significantly decreased in the dentate gyrus on the ipsilateral side compared to sham animals ($P < 0.05$). 6 h post-FPI, NT-3 mRNA levels were significantly decreased in the dentate gyrus on the ispilateral side compared to both the contralateral side ($P < 0.001$) and to sham levels ($P < 0.001$). In addition, NT-3 hybridization levels in the ipsilateral dentate gyrus were significantly lower at 6 h than at 3 h after FPI ($P < 0.05$) (Fig. 4).

trkC mRNA. No differences were revealed either in the film autoradiograms (Fig. 1D, H) or by statistical analysis of densitometric measurements (Fig. 5) in trkC mRNA levels between FPI and sham animals at either 3 or 6 h post-injury.

Discussion

The major finding of this study is that even a mild FPI induces alterations in NTF gene expression in the hippocampus. BDNF mRNA was markedly increased in the granule cells of the dentate gyrus and the CA3 pyramidal cells, and slightly increased in the CA1 region of the hippocampus. Conversely, NT-3 mRNA was significantly decreased in the dentate gyrus. Mild FPI also increased trkB mRNA levels in the dentate gyrus, but did not alter trkC mRNA levels. The overall timing and subregional localization of the acute alterations in NTF and trk mRNA levels are in general agreement with those observed after a moderate FPI (Hicks et al., 1997b; 1998), except for one major difference. Unlike the bilateral changes in NTF gene expression observed in the hippocampus after a FPI of moderate severity (Hicks et al., 1997b;1998), the alterations following a mild injury were restricted to the side of the brain ipsilateral to the impact site. These findings demonstrate that there is a clearly graded response to sham,

mild, and moderate FPI and suggest that some sort of threshold for regulating NTF gene expression may exist. This pattern, unilateral response following mild FPI and bilateral response following moderate FPI, was also characteristic of alterations in immediate early genes (Raghupathi & McIntosh, 1996). These pronounced unilateral alterations induced by a mild FPI may be advantageous for illuminating the regulatory mechanisms and functional consequences of NTF/trk signal transduction pathways, as well as other intracellular signaling pathways.

The coincident increases in mRNA for BDNF and its high-affinity receptor, trkB, in the hippocampus suggests that mild FPI may lead to activation of the BDNF/trkB signal transduction pathways, whereas the decrease in NT-3 mRNA suggests that this pathway may be inactivated. These opposing patterns of BDNF and NT-3 expression have also been observed after a moderate lateral FPI (Hicks et al., 1997), ischemia (Lindvall et al., 1992) and seizures (Gall, 1993), as well as during development (McAllister et al., 1997). The functional consequences of these alterations in NTF gene expression following injury are unknown, but intriguing because of their putative roles in neuroprotection (Beck et al., 1994; Cheng & Mattson 1994; Hayes et al., 1995; Kindy 1993; Kubo et al., 1995; Schabitz et al., 1997; Skaper et al., 1993; Hagg 1997; Lindsay 1993) and learning and memory (Kang & Schuman, 1995; Akeneya et al., 1997; Kang et al., 1997; Huber et al., 1998).

One of the perplexing characteristics of mild head injury is that cognitive deficits often persist despite a lack of pronounced neuropathological alterations. This has been observed both in clinical cases (Blostein et al., 1997; Borczuk, 1997), as well as in rodent models of TBI (Smith et al., 1991; Hogg et al., 1998). Physiological consequences of a

mild FPI include an impairment in long-term potentiation (LTP) in the CA1 region of the hippocampus (Sick et al., 1998). The underlying cause of this impairment is unclear. It has been suggested that less overt changes in the brain following a mild head injury may be responsible for the cognitive and neurophysiological impairments. To this end, recent studies have demonstrated that mild experimental brain injury disrupts cytoskeletal structures such as microtubules (Hicks et al., 1997a) and neurofilaments (Maxwell, 1997), and thus could disrupt neural function without necessarily inducing a fatal injury.

BDNF and NT-3 have both been linked with development of LTP in the hippocampus (Kang & Schuman, 1995; Akeneya et al., 1997; Kang et al., 1997; Huber et al., 1998). Since mild FPI alters NTF mRNA levels in the hippocampus, it is possible that these alterations influence the excitability of neurons. However, the up-regulation of BDNF in the hippocampus following a mild injury would appear to support the generation of LTP, rather than to attenuate it. Alternatively, there is evidence to suggest that the down-regulation of NT-3 mRNA in the dentate gyrus may influence neural plasticity of hippocampal circuits. A study undertaken in NT-3 knock-out mice demonstrated that short term facilitation, but not LTP, was reduced in the hippocampus compared to controls (Kokaia et al., 1998). Whether the decrease in NT-3 mRNA in the granule cells of the dentate gyrus following mild injury is associated with impairments in short term facilitation or LTP in the hippocampus remains to be determined.

In summary, a FPI of mild severity produces unilateral alterations in BDNF, trkB, and NT-3 in the hippocampus. These alterations may underlie some of the cognitive deficits associated with mild head injury. Future studies should be undertaken to evaluate

the effects of modulating NTF levels on recovery of function following mild experimental brain injury.

Acknowledgements

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Figure Legends

Figure 1. Prints of film autoradiograms showing expression of BDNF (A and E), trkB (B and F), NT-3 (C and G), and trkC (D and H) mRNAs in coronal sections from control (sham) rats (A-D) and from rats 6 h following a mild lateral FPI (E-H). Note the increased hybridization for BDNF mRNA in the left dentate gyrus granule cell layer (stratum granulosum; sg) and CA3 hippocampal subfield (E). Note also that trkB hybridization is increased in the left dentate gyrus (F). Conversely, NT-3 hybridization is decreased in the left dentate gyrus (G). The hybridization signal for trkC mRNA in the hippocampus was unaffected by the mild FPI (H).

Figure 2. Regional alterations in BDNF mRNA levels following a mild lateral FPI. Corrected O.D. measurements of hybridization for BDNF mRNA demonstrated a significant increase in the left CA1 region ($P < 0.05$) and CA3 ($P < 0.01$) regions of the hippocampus, and in the ipsilateral dentate gyrus granule cell layer ($P < 0.001$) compared to the contralateral side or to sham injury. There were no significant differences between 3 and 6 h survival periods on BDNF hybridization levels, so the data were combined. Values represent mean ± SEM.

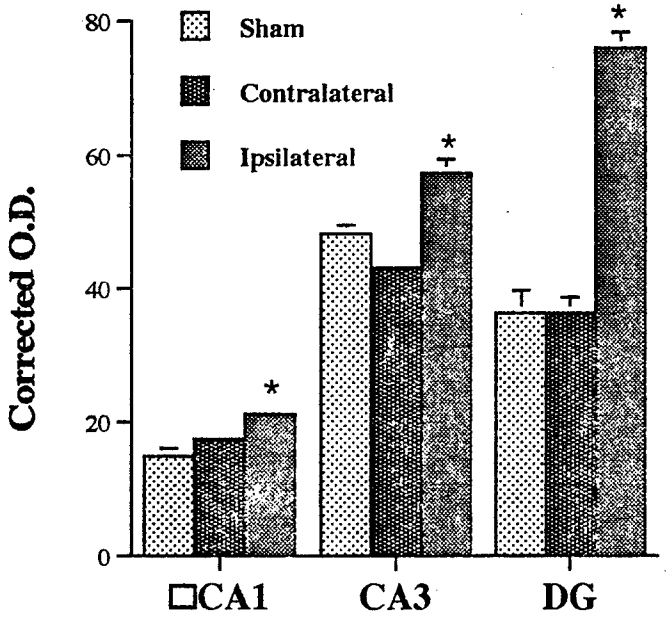
Figure 3. Regional alterations in trkB mRNA levels following a mild lateral FPI. Corrected O.D. measurements of hybridization for trkB mRNA demonstrated a significant increase in the ipsilateral dentate gyrus granule cell layer ($P < 0.001$) compared to the contralateral side or to sham injury. There were no significant

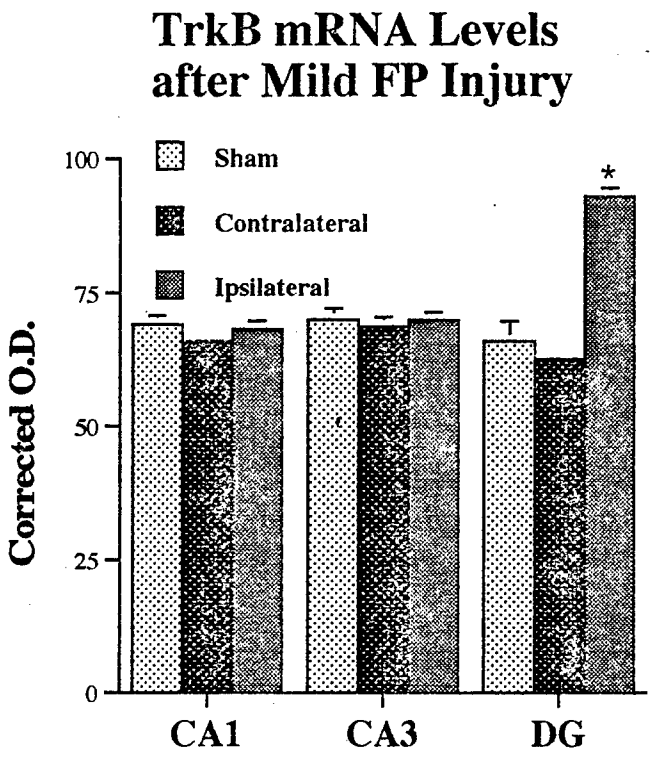
differences between 3 and 6 h survival periods on trkB hybridization levels, so the data were combined. Values represent mean ± SEM.

Figure 4. Regional and temporal alterations in NT-3 mRNA levels following a mild lateral FPI. Corrected O.D. measurements of hybridization for NT-3 mRNA demonstrated a significant decrease in the ipsilateral dentate gyrus granule cell layer 3 h after FPI compared to sham animals ($P < 0.05$). 6 h after FPI, there was a significant decrease on the ipsilateral side compared to the contralateral side ($P < 0.001$) and to sham injury ($P < 0.01$). In addition, the decrease in the left dentate gyrus was greater at 6 h than at 3 h post-FPI ($P < 0.05$). Values represent mean ± SEM.

Figure 5. Regional alterations in trkC mRNA levels were not observed following a mild lateral FPI. There were no significant differences between 3 and 6 h survival periods on trkC hybridization levels, so the data were combined. Values represent mean ± SEM.

BDNF mRNA Levels after Mild FP Injury




TrkB mRNA Levels
after Mild FP Injury
Sham
Contralateral
Ipsilateral
Corrected O.D.
100
75
50
25
0
*
CA1
CA3
DG

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
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